Graphitized Carbon Black Enrichment and UHPLC-MS/MS Allow to Meet the Challenge of Small Chain Peptidomics in Urine

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The Supporting Information contains an integration to the experimental section with details about calculation of recoveries, matrix effect and process efficiency, a general figure for GCB SPE protocol (Figure S1), results of the optimization experiments of the GCB procedure from standards (Table S1), list of peptide standards with related mass, GRAVY value, molecular weight, pI and MRM acquisition conditions (Table S2), plots showing the

trend of standard peptide recoveries under the 6 procedures described in Table S2 as a function of GRAVY, MW and pI (Figure S2), results on the performance of the final GCB SPE method from spiked urine samples (Table S3), plots showing the trend of standard peptide recoveries under the final procedure as a function of GRAVY value, MW and pI for standards in solvent and spiked urine samples (Figure S3), figure on the reproducibility of the chromatographic separation by iHILIC Fusion with chromatograms, boxplot of $\log_2$(area) and multiscatterplots with Pearson correlation coefficient (Figure S4).

Integration to section "Preparation of Urine Samples and Peptide Extraction":

calculation of recovery, matrix effect and process efficiency.

Recovery, matrix effect and process efficiency were calculated from three sets of experiments, namely urine samples spiked with the standard peptides before (set 1) and after (set 2) the GCB enrichment and clean-up, and neat standard solutions (set 3). Absolute peak areas (i.e., without normalization) were measured and used in the following equations.

Recovery (RE%) was calculated using equation 1:

$$RE\% = (Area_{set1}/Area_{set2}) \times 100 \qquad (1)$$

Matrix effect (ME%) was calculated using equation 2:

$$ME\% = (Area_{set2}/Area_{set3}) \times 100 \qquad (2)$$

The overall process efficiency (PE%) can be obtained by applying one of the two following equations:

$$PE\% = (Area_{set1}/Area_{set3}) \times 100 \qquad (3a)$$

$$PE\% = RE \times ME \quad . \qquad (3b)$$

The experiments were performed using 100 ng of each standard peptide.

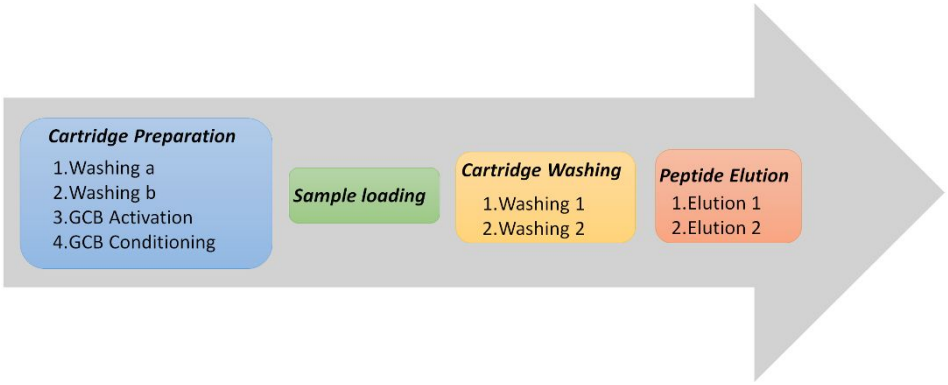

Figure S1. Optimization protocol for GCB applied to short peptide recovery from standard solutions.

Table S1. Test with standard peptide mixture. Ten mL of loading buffer (LB) were spiked with 100 ng of each standard peptide. The general protocol and names of each step are reported in Figure S1. The detailed composition of the loading buffer (LB), washing buffer (WB) and elution buffer (EB) is reported below the table.

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**	**Exp 5[a]**	**Exp 6**	**Urine[b]**
GCB Amount	250 mg	250 mg	250 mg	250 mg	250 mg	250 mg	500 mg
Washing a	5 mL EB2	5 mL EB2	5 mL EB3	5 mL EB4	5 mL EB5	5 mL EB6	5 mL EB5
Washing b	5 mL EB1	5 mL EB1	5 mL EB1	5 mL EB1	5 mL WB2	5 mL WB2	5 mL WB2
GCB Activation	10 mL LB1	10 mL LB1	10 mL LB1	10 mL LB1	10 mL LB1	10 mL LB1	10 mL LB1
GCB Conditioning	10 mL LB2	-	10 mL LB3	10 mL LB3	10 mL LB3	10 mL LB3	10 mL LB3
Sample Loading	10 mL LB2	10 mL LB1	10 mL LB3	10 mL LB3	10 mL LB3	10 mL LB3	2 mL urine + 8 mL LB3
Washing 1	2 mL LB2	2 mL LB1	2 mL LB3	2 mL LB3	2 mL LB3	2 mL LB3	2 mL LB3
Washing 2	-	-	-	0.5 mL WB1	0.5 mL WB1	0.5 mL WB1	0.5 mL WB1
Elution 1	5 mL EB1	5 mL EB1	5 mL EB1	10 mL EB4	10 mL EB5	10 mL EB6	10 mL EB5
Elution 2	10 mL EB2	10 mL EB2	10 mL EB3	-	-	-	-
Backflushing elution	No	No	No	Yes	Yes	Yes	Yes
Recovery (%)	12-93%	15-100%	32-99%	61-99%	61-100%	61-97%	60-100%
RSD (%)	10-18%	9-15%	13-22%	15-35%	8-17%	14-23%	9-17%

LB1: H_2O with 0.1 mol L^{-1} HCl

LB2: H_2O with 50 mmol L^{-1} HCOOH

LB3: H_2O 20 mmol L^{-1} TFA

WB1: MeOH

WB2: MeOH 20 mmol L^{-1} TFA

EB1: MeOH 50 mmol L^{-1} HCOOH

EB2: CH_2Cl_2/MeOH, 80:20 (*v*/*v*) 50 mmol L^{-1} HCOOH

EB3: CH_2Cl_2/MeOH, 80:20 (*v*/*v*) 10 mmol L^{-1} TMAC

EB4: CH_2Cl_2/MeOH, 80:20 (*v*/*v*) 10 mmol L^{-1} TMAC 50 mmol L^{-1} HCOOH

EB5: CH_2Cl_2/MeOH, 80:20 (*v*/*v*) 20 mmol L^{-1} TFA

EB6: CH_2Cl_2/MeOH, 80:20 (*v*/*v*) 50 mmol L^{-1} TFA

[a]final conditions for pure solvents

[b]final conditions for urine

Table S2. List of peptide standards with related mass, Grand average of hydropathicity (GRAVY) value, molecular weight (MW), isoelectric point (pI) and MRM acquisition conditions (*m*/*z* of precursor ions and product ions, collision energy and S-lens values).

Peptide	MW	GRAVY index	pI	Precursor ion (*m*/*z*) $[M+H]^+$	Product ions (*m*/*z*)	Collision energy (eV)	S-lens (V)
KH	283.1644	-3.55	8.75	285	110	28	96
				285	156	14	96
PL	228.1474	1.1	5.95	229	68	37	80
				229	70	19	80
PI	228.1474	1.45	5.95	229	68	37	80
				229	70	19	80
LP	228.1474	1.1	5.95	229	86	32	70
				229	116	16	70
SH	242.1015	-2	6.45	243	110	25	80
				243	156	15	80
KHK	411.2594	-3.67	10.00	412	110	34	100
				412	231	25	100
IPI	341.2315	2.47	5.53	342	70.5	31	100
				342	229	17	100
LPL	341.2315	2	5.52	342	70.5	31	100
				342	229	17	100
RF	321.1901	-0.85	9.75	323	70	33	129
				323	175	20	129
VEP	343.1743	-0.3	3.99	344	116	14	87
				344	229	13	87
VRGP	427.2543	-0.58	9.72	428	173	25	150
				428	239	27	150
GDLE	432.1856	-0.9	4.08	433	86	23	113
				433	261	19	113
IPPL	438.2842	1.28	5.52	439	211	17	111
				439	326	19	111

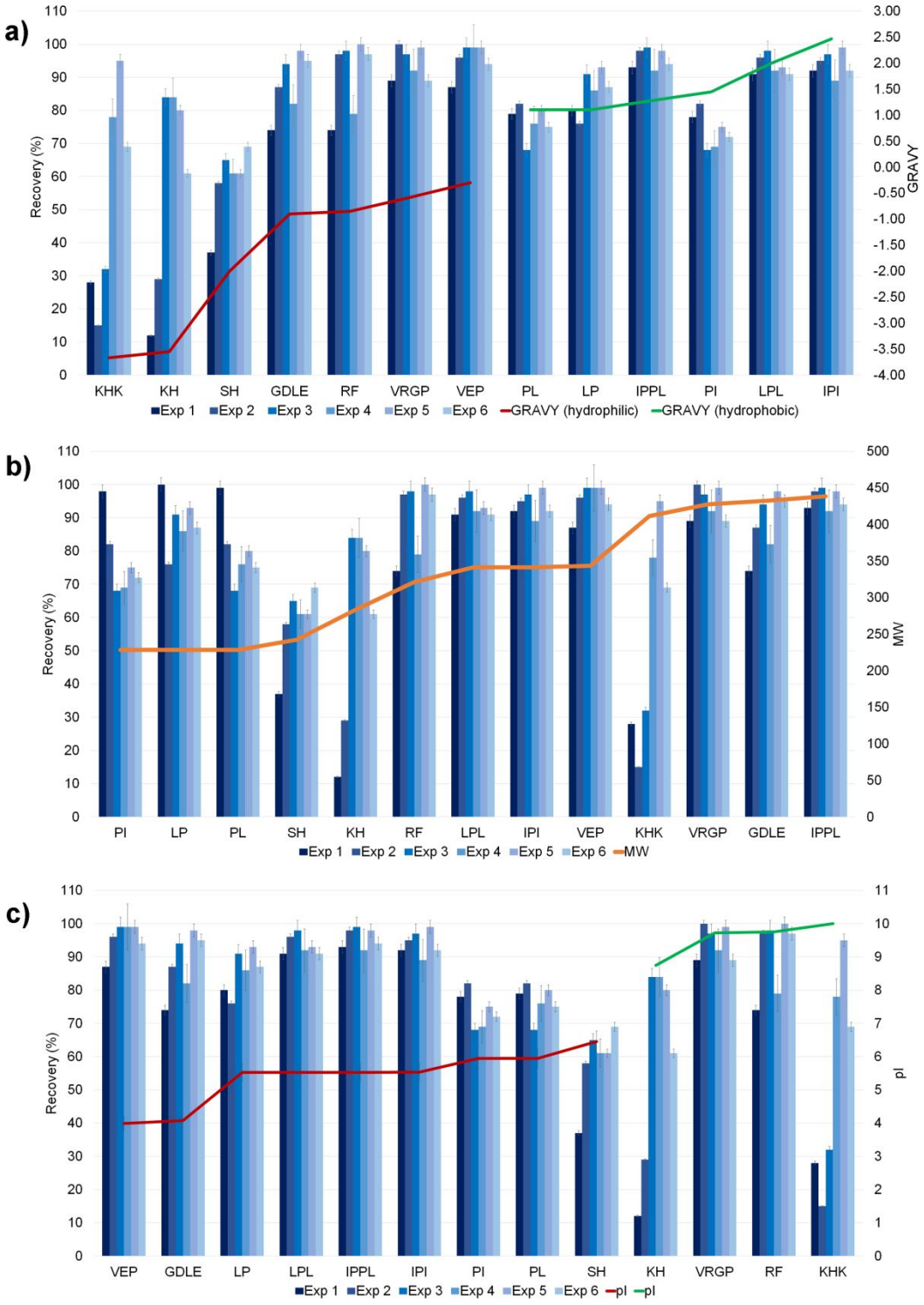

Figure S2. Plots showing the trend of standard peptide recoveries under the 6 procedures described in Table S2 as a function of GRAVY value (a), MW (b) and pI (c).

Table S3. Method performance of the GCB clean-up of short peptides from spiked urine samples.

	Recovery (%)	Matrix effect (%)	Process efficiency (%)
KH	76%	96%	73%
PL	75%	115%	86%
PI	75%	111%	83%
LP	87%	109%	95%
SH	60%	85%	51%
KHK	93%	90%	84%
IPI	94%	88%	83%
LPL	87%	86%	75%
RF	100%	102%	102%
VEP	91%	116%	106%
VRGP	99%	114%	113%
GDLE	90%	114%	103%
IPPL	89%	103%	92%

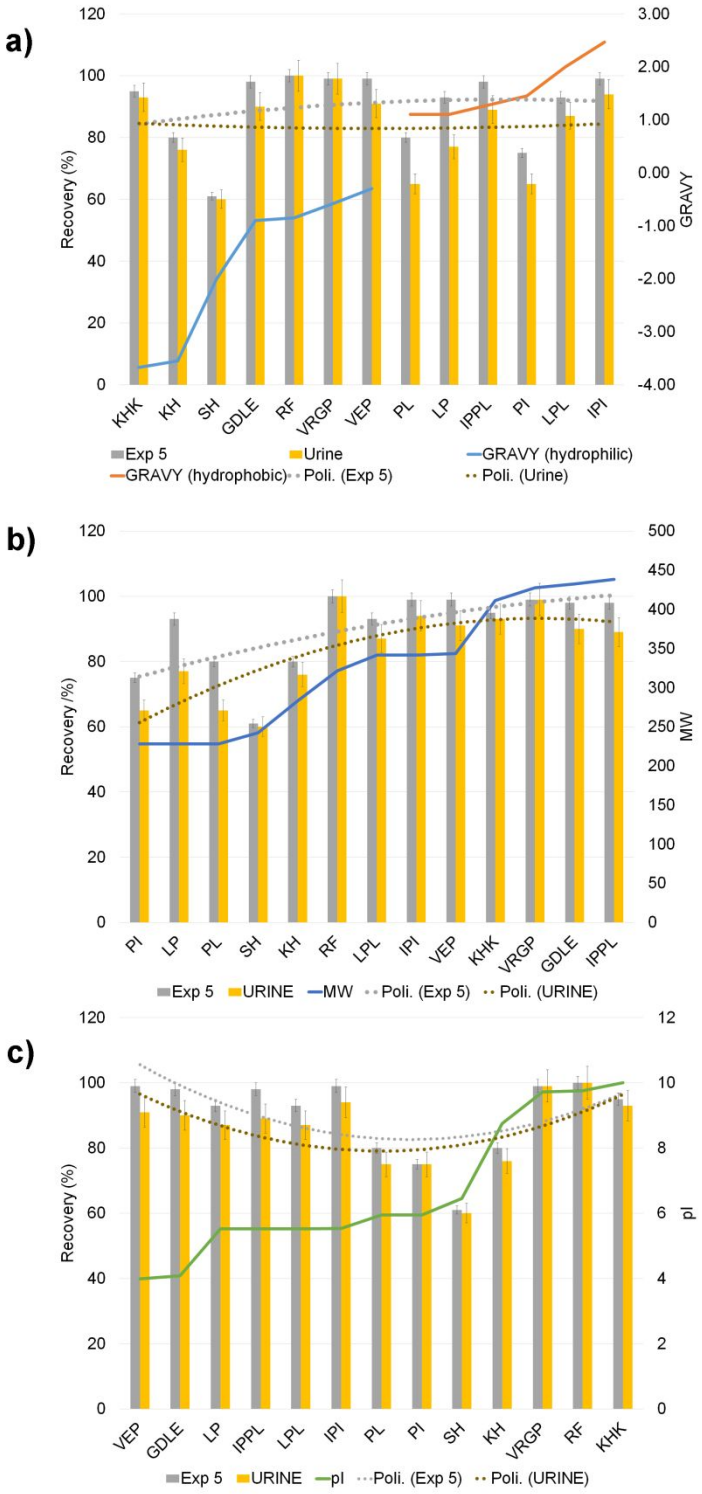

Figure S3. Plots showing the trend of standard peptide recoveries under the final procedure described in Table S2 as a function of GRAVY value (a), MW (b) and pI (c) for standards in solvent (Experiment 5) and spiked urine samples.

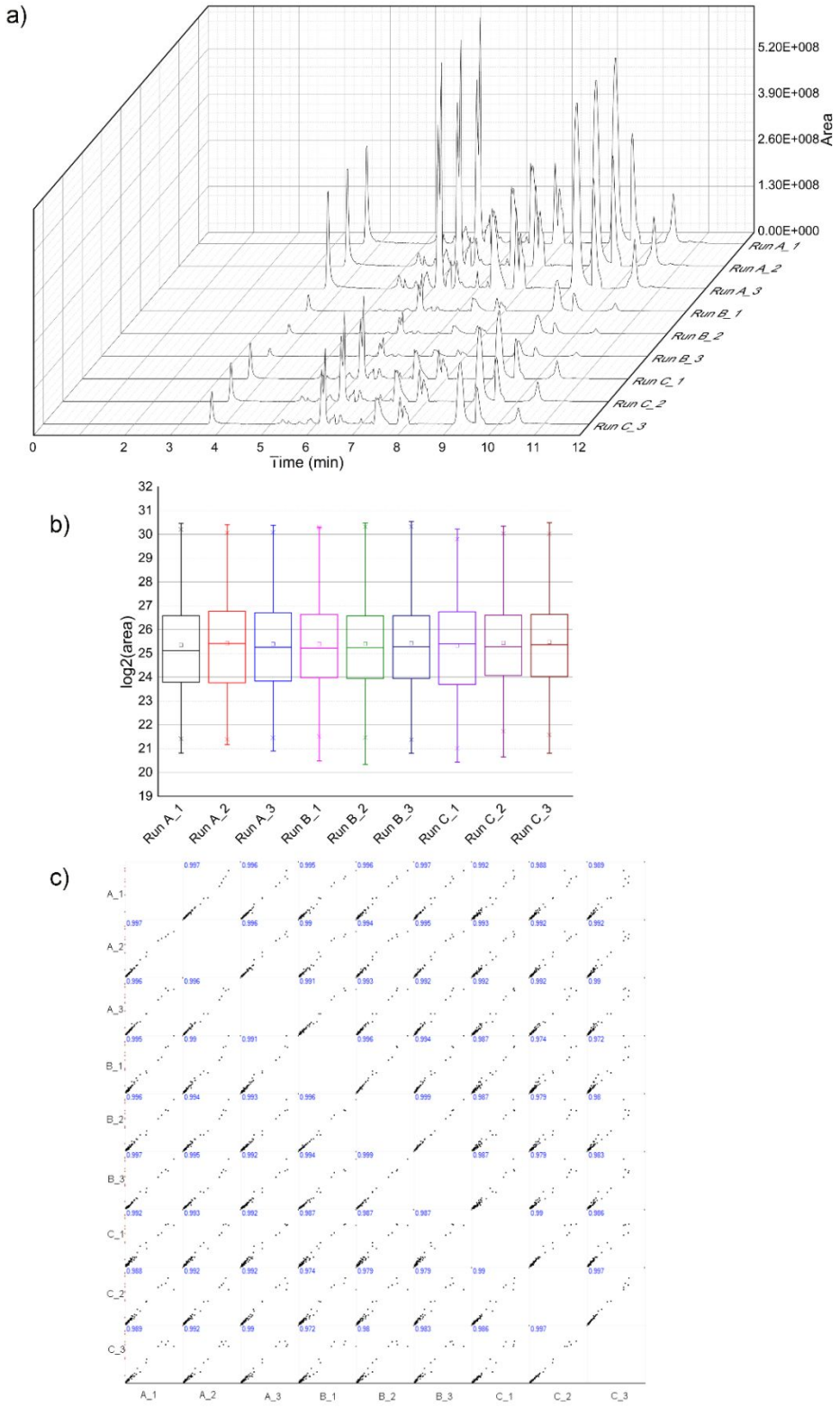

Figure S4. Reproducibility of the chromatographic separation by iHILIC Fusion. Chromatograms (a) and $\log_2$(area) (b) of the identified peptides across the three technical replicates for each experimental replicate. Multiscatterplots for HILIC runs with Pearson correlation coefficient (c).